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ANTI-PROLIFERATIVE EFFECT OF RADIOLABELLED OCTREOTIDE IN A METASTASES MODEL IN RAT LIVER

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Most neuroendocrine tumours and several other tumours, such as breast carcinoma and malignant lymphoma, express somatostatin receptors (SS-Rs). Lesions expressing these receptors can be visualised by receptor scintigraphy using a low radioactive dose of the radiolabelled SS analogue [^{111}In-DTPA^{0}]octreotide. This radioligand is internalised and transported to the lysosomes with a long residence time of ^{111}In. The aim of this experimental study in rats was to investigate whether the same agent, given in a high radioactive dose, can be used for therapy of hepatic metastases of different tumour cell lines. The development of hepatic metastases was determined 21 days after direct injection of SS-R-positive or -negative tumour cells into the vena porta in rats. On day 1 and/or 8, animals were treated with 370 MBq (0.5 µg) [^{111}In-DTPA^{0}]octreotide. In one experiment, using SS-R-positive tumour cells, animals were pre-treated with a high dose of cold octreotide to block the SS-R by saturation. The number of SS-R-positive liver metastases was significantly decreased after treatment with [^{111}In-DTPA^{0}]octreotide. Blocking the SS-R by octreotide substantially decreased the efficacy of treatment with [^{111}In-DTPA^{0}]octreotide, suggesting that the presence of SS-R is mandatory. This was confirmed by the finding that the number of SS-R-negative liver metastases was not affected by treatment with [^{111}In-DTPA^{0}]octreotide. Therefore, we conclude that *(i)* high radioactive doses of [^{111}In-DTPA^{0}]octreotide for PRRT (peptide receptor radionuclide therapy) can inhibit the growth of SS-R-positive liver metastases in an animal model, *(ii)* PRRT is effective only if SS-Rs are present on the tumours, *(iii)* the effect of PRRT with [^{111}In-DTPA^{0}]octreotide can be reduced by pre-treatment with cold octreotide, which indicates that receptor binding is essential for PRRT. Our data suggest that PRRT with radiolabelled octreotide might be a new promising treatment modality for SS-R-positive tumours. *Int. J. Cancer* 81:767–771, 1999.
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Somatostatin (SS) is a small regulatory peptide which inhibits the release of various hormones and may act as neurotransmitter in the CNS (Reichlin, 1983). A number of observations have also suggested an anti-proliferative effect of SS and its analogues (Lamberts *et al*., 1991; Kvols *et al*., 1986; Schally, 1988). Critical to these actions of SS is the presence of somatostatin receptors (SS-Rs). SS-Rs have been demonstrated on a variety of human tumours and their metastases (Reubi *et al*., 1992*b*). At least 5 different human SS-R subtypes (sst_{1-5}) have been cloned (Kubota *et al*., 1994). All subtypes bind SS with high affinity, while their affinity for the SS analogue octreotide differs considerably. Octreotide binds with high affinity to sst_2 and sst_5 and to a lesser degree sst_3, while no binding to sst_1 or sst_4 occurs. However, the vast majority of human SS-R-positive tumours express sst_2 (Hofland *et al*., 1997). After injection of [^{111}In-DTPA^{0}]octreotide, SS-R-positive tumours show uptake of radioactivity, which can be visualised with a gamma camera (Krenning *et al*., 1989, 1993). ^{111}In emits not only gamma rays, which can be visualised, but also internal-conversion and Auger electrons with a medium to short tissue penetration (200–550 µm, 0.02–10 µm, respectively) (Bambyrek *et al*., 1972; Howell, 1992; Adelstein, 1993). *In vivo*, ^{111}In is internalised and transported into the lysosomes of SS-R-positive cells after administration of [^{111}In-DTPA^{0}]octreotide (Duncan *et al*., 1997) with a long residence time in the tumour (biological half-life >700 hr) (Krenning *et al*., 1994). Therefore, an effect on tumour cell proliferation by these electrons may be expected. Inspired by the ample knowledge of [^{111}In-DTPA^{0}]octreotide scintigraphy, peptide receptor radionuclide therapy (PRRT) with high doses of radioactivity labelled with this SS analogue has been initiated clinically (Fjälling *et al*., 1996; Krenning *et al*., 1994, 1996). In addition, 3 experimental studies demonstrated tumour growth-inhibitory effects on solid s.c. tumours by radiolabelled SS analogues in animal models (Zamora *et al*.,1996; Stolz *et al*., 1996, 1998). However, this new concept of SS-R-mediated PRRT has never been tested for the treatment of SS-R-positive liver metastases with an ^{111}In-labelled SS analogue. In a previous report, we demonstrated that growth of SS-R-positive CA20948 pancreatic tumour cells in the liver could be inhibited by s.c. administration of 15 µg cold octreotide twice per day, whereas no effect was achieved with SS-R-negative CC-531 colon carcinoma cells (Van Eijck *et al*., 1994). Using the same *in vivo* tumour models, we describe here the marked SS-R-dependent efficacy of PRRT with [^{111}In-DTPA^{0}]octreotide to inhibit the development of SS-R-positive liver metastases.

MATERIAL AND METHODS

Animals

Male rats of the inbred WAG and Lewis strains, 10 to 14 weeks old and 225 to 250 g, were obtained from Harlan-CPB (Austerlitz, The Netherlands). Animals were kept under standard laboratory conditions (12 hr light/12 hr dark) and given a standard laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to the rules of the Dutch Animal Experimentation Act and was approved by the Committee on Animal Research of Erasmus University.

Tumours

The pancreatic tumour CA20948 was originally induced by azaserine. This SS-R-positive tumour is of acinar origin and is transplantable in syngeneic Lewis rats. The tumour was transplanted and maintained in the liver after direct injection into the vena porta. To produce artificial liver metastases, tumours were excised from donor livers, cleaned from normal liver tissue and pressed through sieves with decreasing mesh size. The resulting suspension was washed twice in RPMI 1640. Viability was measured with Trypan blue exclusion (0.3% in a 0.9% NaCl solution). A suspension of 2.5×10^6 living cells/ml was used for direct injection into the vena porta.

Tumour CC-531 is an SS-R-negative, 1,2-dimethylhydrazine-induced, moderately differentiated colon adenocarcinoma, transplantable in syngeneic WAG rats. The tumour is maintained in tissue culture as a monolayer in RPMI 1640 medium (GIBCO, Paisley, UK) supplemented with 5% FCS. Cells were harvested from stationary cultures by gentle trypsinisation. A suspension of

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2.5×10^6 living cells/ml was used for direct injection into the vena porta.

The presence/absence of the SS-R on both tumour cell lines was determined by specific binding of [^{125}I-Tyr^3]octreotide. Binding to membrane preparations of CA20948 pancreatic tumour cells (IC_{50} 0.6 nM and B_{max} 110 fmol/mg membrane protein), but not to the membrane preparations of the CC-531 colon tumour cells, was demonstrated (Van Eijck *et al*., 1994).

Radiolabelling and quality control of the radioligand

[DTPA^0]octreotide (Pentetreotide, DRN 4920) and $^{111}InCl_3$ (DRN 4901, 370 MBq/ml in HCl, pH 1.5 to 1.9) were obtained from Mallinckrodt (Petten, The Netherlands). Octreotide was a gift of Sandoz (Basle, Switzerland). Labelling was performed by diluting freeze-dried [DTPA^0]octreotide in 1 ml saline and adding this to the $^{111}InCl_3$. Thirty minutes after the start of this procedure, quality control was performed by instant thin-layer chromatography with silica gel and 0.1 M sodium citrate, pH 5, as eluent (Bakker *et al*., 1991). The labelling efficiency of [^{111}In-DTPA^0]octreotide was over 98%. Each administration of the radioligand consisted of 370 MBq ^{111}In labelled with 0.5 µg [DTPA^0]octreotide, referred as 370 MBq (0.5 µg) [^{111}In-DTPA^0]octreotide.

Experimental procedure

Under ether anesthesia, the abdomen was opened through a 2.5-cm midline incision. Then, 0.5×10^6 viable SS-R-positive CA20948 cells in 0.2 ml RPMI 1640 were injected slowly into the vena porta through a $0.4 \times$ 12-mm needle. The abdominal wall was closed in one layer by a continuous silk suture. The day after the operation, rats were randomised into experimental and control groups. Each group consisted of 5 or 6 rats.

In experiment 1, rats in the experimental group were treated with 370 MBq (0.5 µg) [^{111}In-DTPA^0]octreotide i.v. on days 1 and 8. Rats in the control group were injected with vehicle, 0.5 µg [DTPA^0]octreotide i.v. In experiment 2, 2 groups were added, in which treatment was given on day 1 or 8 alone. Experiment 3 was designed to investigate the effect of pre-treatment with an SS-R-blocking concentration of octreotide. The effect of 370 MBq (0.5 µg) [^{111}In-DTPA^0]octreotide i.v. on days 1 and 8 was studied with and without SS-R blocking. In the group with receptor blocking, rats were treated with s.c. administration of 1 mg octreotide 30 min. prior to injection of the radioligand (Breeman *et al*., 1994). Another 6 rats were injected with 1 mg octreotide s.c. only, for comparison. In experiment 4, the importance of the SS-R in PRRT was investigated. In this experiment, 0.5×10^6 SS-R negative CC-531 cells in 0.2 ml RPMI were injected into the vena porta. Treatment was given according to the same schedule as used in experiment 1.

All rats were killed 21 days after inoculation of tumour cells. Livers were removed and immersed in PBS. Tumour growth was determined by 2 investigators counting the number of metastases on the surface of the liver lobes (up to 100) while blinded for treatment modality.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney U-test on categorised outcomes and Fisher's exact test on proportions. Statistical significance was defined as $p < 0.05$.

RESULTS

In experiment 1, PRRT with administration of 370 MBq (0.5 µg) [^{111}In-DTPA^0]octreotide on days 1 and 8 induced a significant ($p < 0.01$) decrease in the number of hepatic metastases 21 days after direct injection of CA20948 pancreatic SS-R-positive tumour cells into the vena porta. In the control group, all animals showed tumour colonies in the liver. After PRRT, tumour colonies were found in only 2 of 6 animals (Table I). Examples of livers from both experimental and control groups are shown in Figure 1.

Table II shows the results of experiment 2, in which 2 groups were added and treated on day 1 or on day 8 only. Treatment on day 1 or 8 alone also decreased tumour load significantly ($p < 0.01$), while no differences could be found between treatment on either day. Treatment on both days, however, resulted in lesser liver colonies than when a single treatment was given ($p < 0.05$). In the third experiment (Table III), PRRT again induced a significant decrease in the number of tumour colonies ($p < 0.01$). Pre-treatment with 1 mg octreotide prior to PRRT resulted in a significantly higher number of liver tumour colonies compared to PRRT without receptor blocking ($p < 0.01$). In a third group that was treated only with octreotide without PRRT, all rat livers contained over 100 colonies.

TABLE I – EFFECT OF PRRT ON SS-R-POSITIVE LIVER METASTASES

Treatment	Number of animals with 0 to 100 metastases			
	0	1–20	21–100	>100
Controls	—	2	2	2
[^{111}In-DTPA^0]octreotide	4	2	—	—

Number of animals with given range of metastases, 21 days after direct injection of SS-R-positive CA20948 tumour cells into the vena porta. The effect of PRRT on days 1 and 8 with 370 MBq (0.5 µg) [^{111}In-DTPA^0]octreotide is significantly different ($p < 0.01$) from that of treatment with 0.5 µg cold [DTPA^0]octreotide (controls).

The results of PRRT on the formation of liver metastases after direct injection of SS-R-negative CC-531 tumour cells into the vena porta are shown in Table IV. No difference in the number of liver tumour colonies was found between the experimental group and the control group.

DISCUSSION

Several studies have demonstrated that non-radioactive SS analogues inhibit tumour growth in experimental tumour models (Smith and Solomon, 1988; Nott *et al*., 1989; Schally, 1988). Most tumours used in these experiments contain SS-Rs and were studied *in vitro* and *in vivo* as a transplantable tumour. Possible mechanisms of the anti-proliferative effects of these non-radioactive analogues include stimulation of hepatic reticuloendothelial system activity, reduction of portal venous flow and interference with secretion of growth factors, such as prolactin, growth hormone and insulin-like growth factor-I (Lamberts *et al*., 1991). Results of our previous studies, however, indicated that tumour growth inhibition by octreotide was mediated *via* SS-Rs since no inhibition of tumour growth could be found when SS-R-negative tumour cells were used (Van Eijck *et al*., 1994). To demonstrate the presence of SS-R on tumour cells, *in vivo* peptide receptor scintigraphy with radiolabelled SS analogues, such as [^{111}In-DTPA^0]octreotide, has been proven to be a sensitive and specific technique. Using this technique, primary tumours and their, often unrecognised, metastases of a wide variety of human SS-R-positive types can be localised (Krenning *et al*., 1993).

When PRRT with radiolabelled octreotide is considered, it is important to know whether octreotide has a high affinity for SS-Rs expressed on the tumour cells and whether the radioligand is actively internalised by the same cells. Five different SS-R subtypes (sst_{1-5}) have been cloned and characterised on tumour cells, which show a distinct pharmacological binding profile to octreotide. Octreotide was found to bind with high affinity to sst_2 and sst_5 only and showed a low or no affinity to sst_3, sst_1 and sst_4. SS-R subtype expression in different types of human cancer has now been studied using *in situ* hybridisation, RNAse protection assays and RT-PCR (Reubi *et al*., 1992*a*; Kubota *et al*., 1994; Hofland *et al*., 1995*b*, 1997; Vikic-Topic *et al*., 1995). These results indicate that most tumours express multiple SS-R subtypes at the same time. In the vast majority of human SS-R-positive tumours, however, sst_2 is predominantly expressed. Since several *in vivo* and *in vitro* studies have demonstrated that [^{111}In-DTPA^0]octreotide retains high affinity to sst_2, this compound might be suitable for PRRT. The internalisation by tumour cells of the radioligand is

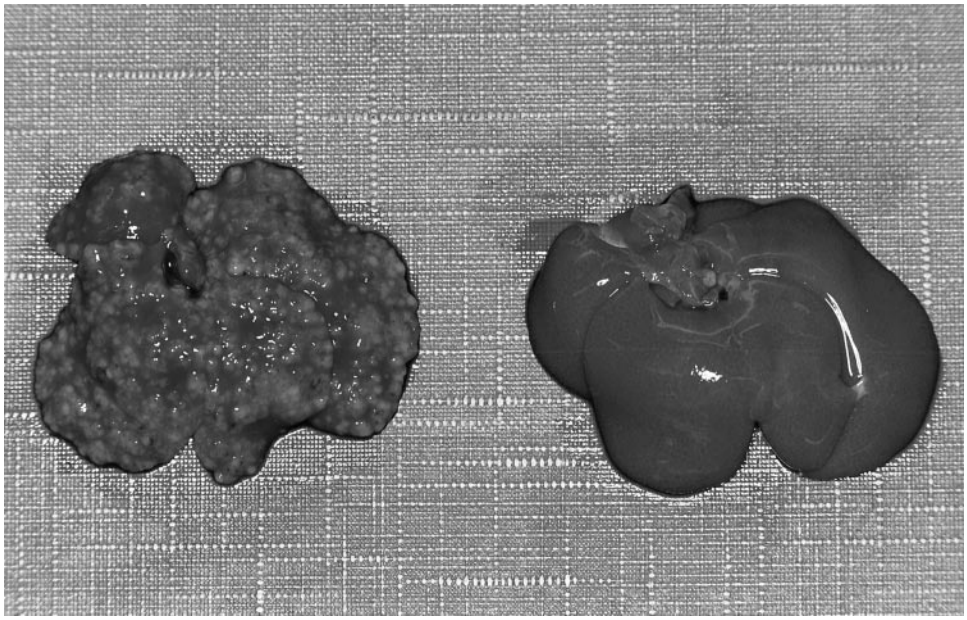

FIGURE 1 – Effect of PRRT on SS-R-positive liver metastases. Livers from the control and experimental groups (left and right, respectively); data presented in Table I. Left: Liver with >100 metastases (control). Right: Liver with no visible metastases after PRRT on days 1 and 8 with 370 MBq (0.5 µg) [^{111}In-DTPA^{0}]octreotide.

TABLE II – EFFECT OF PRRT ON SS-R-POSITIVE LIVER METASTASES, GIVEN AT DIFFERENT TIMES

Treatment	Number of animals with 0 to 100 metastases			
	0	1–20	21–100	>100
Controls	—	—	—	6
[^{111}In-DTPA^{0}]octreotide day 1	—	4	1	—
[^{111}In-DTPA^{0}]octreotide day 8	—	3	2	—
[^{111}In-DTPA^{0}]octreotide days 1 and 8	3	2	—	—

Number of animals with given range of metastases (5 or 6 animals per group), 21 days after direct injection of SS-R-positive CA20948 tumour cells into the vena porta. PRRT with 370 MBq (0.5 µg) [^{111}In-DTPA^{0}]octreotide was given on day 1 or 8 or on both days 1 and 8. The effect of all treatment schedules was significantly different ($p < 0.01$) from that of 0.5 µg cold [DTPA^{0}]octreotide on days 1 and 8 (controls). Treatment on days 1 and 8 was significantly different from treatment on day 8 alone ($p < 0.05$). No significant difference was found between the effect of treatment on day 8 or day 1.

TABLE III – EFFECT OF PRE-BLOCKING OF THE SS-R WITH OCTREOTIDE ON PRRT OF SS-R-POSITIVE LIVER METASTASES

Treatment	Number of animals with 0 to 100 metastases			
	0	1–20	21–100	>100
[^{111}In-DTPA^{0}]octreotide	3	3	—	—
[^{111}In-DTPA^{0}]octreotide + blocking with octreotide	—	—	4	1
Octreotide	—	—	—	6

Number of animals with given range of metastases, 21 days after direct injection of SS-R-positive CA20948 tumour cells into the vena porta. Effect of PRRT on days 1 and 8 with 370 MBq (0.5 µg) [^{111}In-DTPA^{0}]octreotide without or with 1 mg octreotide s.c. to block the SS-R. A third group of rats was used as control to investigate the effect of 1 mg octreotide s.c. Group 1 *vs.* group 3, $p < 0.01$; group 1 *vs.* group 2, $p < 0.01$; group 2 *vs.* group 3, $p < 0.05$.

TABLE IV – EFFECT OF PRRT ON SS-R-NEGATIVE LIVER METASTASES

Treatment	Number of animals with 0 to 100 metastases			
	0	1–20	21–100	>100
Controls	—	—	3	3
[^{111}In-DTPA^{0}]octreotide	—	—	2	4

Number of animals with given range of metastases, 21 days after direct injection of SS-R-negative CC-531 tumour cells into the vena porta. The effect of PRRT on days 1 and 8 with 370 MBq (0.5 µg) [^{111}In-DTPA^{0}]octreotide is not significantly different from that of treatment with 0.5 µg cold [DTPA^{0}]octreotide (controls).

another important aspect of PRRT with radiolabelled octreotide. Internalisation by receptor-mediated endocytosis may bring the radionuclide closer to its target, the DNA of the tumour cell. Hofland *et al*. (1995*a*) showed that the radio-iodinated [Tyr^{3}]octreotide is rapidly internalised by mouse AtT20 pituitary tumour cells and by primary cultures of human GH-secreting pituitary tumour cells. Duncan *et al*. (1997) reported internalisation of [^{111}In-DTPA^{0}]octreotide *in vivo* into the lysosomes. In agreement with these studies, Andersson *et al*. (1996) demonstrated internalisation of ^{111}In into human carcinoid and gastrinoma cells after incubation with [^{111}In-DTPA^{0}]octreotide. PRRT with high doses of [^{111}In-DTPA^{0}]octreotide might therefore be effective because of the emission of Auger and internal-conversion electrons by ^{111}In (Howell, 1992; Adelstein, 1993). Due to internalisation of [^{111}In-DTPA^{0}]octreotide, the radiotoxicity of these electrons is high since the DNA of the cell is within the micrometer range from the internalised radionuclide.

We investigated the anti-proliferative effect of [^{111}In-DTPA^{0}]octreotide on SS-R-positive and -negative tumour cells in a rat liver metastases model. The major finding from these experiments is that PRRT with [^{111}In-DTPA^{0}]octreotide leads to a marked inhibition of intrahepatic growth of SS-R-positive tumour cell colonies. Furthermore, in repeated experiments, most treated animals showed no SS-R-positive tumour colonies, an outcome that was not observed in earlier experiments using non-radioactive octreotide (van Eijck *et al*., 1994). Possibly, the reduced number of tumour colonies could be due to interference with either adherence or growth of the tumour cells when therapy was given on day 1, but treatment given 8 days after tumour cell inoculation also led to a significant tumour growth inhibitory effect.

The results of the present experiments suggest that tumour growth inhibition was predominantly due to the specific binding of [^{111}In-DTPA^{0}]octreotide to SS-Rs and not to a systemic or a secondary mechanism, since PRRT had no effect on SS-R-negative tumour cells. Furthermore, pre-treatment with 1 mg octreotide, resulting in saturation of the SS-Rs, led to a diminished effect of PRRT. The finding that blocking the SS-Rs did not abolish the therapeutic effect of PRRT may be due to the inability to completely block all of the receptors by pre-treatment since there is a competitive equilibrium (Smith and Solomon, 1988; De Jong *et al*., 1995). The presence or absence of the SS-R is not the only difference between the models; however, no data are available on differences in radiosensitivity.

^{111}In, however, is not the optimal radionuclide for PRRT since it lacks the higher energies of α and β particles: the β-particle emitter ^{90}Y, with a maximum β-energy of 2.3 MeV and a half-life of 64 hr, may be more suitable. Since ^{90}Y-DTPA is not stable *in vivo*, octreotide has been derivatized with the DOTA (tetraazacyclododecanetetraacetic acid) chelator for stable radiolabelling with ^{90}Y. This resulted in the radioligand [^{90}Y-DOTA^{0},D-Phe^{1},Tyr^{3}]octreotide; a single i.p. injection of 500 μCi [^{90}Y-DOTA^{0},D-Phe^{1},Tyr^{3}]octreotide led to a significant decrease (25%) in the size of SS-R-positive AR42J pancreatic tumours in nude mice (Stolz *et al*., 1996). Stolz *et al*. (1998) also reported the curative potential of [^{90}Y-DOTA^{0},D-Phe^{1},Tyr^{3}]octreotide for the SS-R-positive tumour CA20948 inoculated into the hindlegs of Lewis rats, the presence of sst_2 and sst_5 being reported for this model. Clinical phase I studies with this radioligand in patients with neuroendocrine tumours have already started. A third experimental study similarly demonstrated an effect on tumour growth by radiolabelled analogues in animal models. Multiple intra-tumour injections of ^{188}Re-labelled RC-160, another SS analogue, resulted in reduction of tumour size and prolonged survival in nude mice bearing positive PC-3 human prostatic adenocarcinomas (Zamora *et al*., 1996). PRRT of SS-R-positive tumours with [^{111}In-DTPA^{0}]octreotide has also been carried out in several patients with inoperable, metastasised neuroendocrine tumours. After multiple high doses of [^{111}In-DTPA^{0}]octreotide radioactivity (up to 53 GBq), impressive effects on hormone production and a decrease in tumour volume have been observed (Fjälling *et al*., 1996; Krenning *et al*., 1994, 1996; Wiseman and Kvols, 1995).

Typical radiation doses to tissues with administered doses of 6,000 to 7,000 MBq [^{111}In-DTPA^{0}]octreotide are as follows: kidneys 300 to 1,400 cGy [depending on the relative biological effectiveness (RBE, 1 to 20) for Auger electrons], spleen 20 cGy, liver 50 cGy, bone marrow 13 cGy, thyroid gland 25 cGy and pituitary 70 cGy (Krenning *et al.*, 1992).

In addition, within administrated doses of 6,000 to 7,000 MBq, the estimated/calculated tumour radiation doses are, for a 10-g tumour (assumptions: 1% uptake, effective half-life equal to the physical half-life), 1,700 and 6,700 cGy (RBE for Auger electrons 1 and 20, respectively) and, for a 100-g tumour (1 % uptake), 250 and 750 cGy, respectively (Krenning *et al*., 1998). We are currently also performing toxicology studies after 370 MBq (0.5 μg) [^{111}In-DTPA^{0}]octreotide in control rats not bearing tumours.

Since we do realise that tumour load in our experiments was relatively small and the doses used relatively high, more PRRT experiments, in more advanced stages of tumour development and with different doses of [^{111}In-DTPA^{0}]octreotide, are necessary. However, our present results suggest that PRRT may be a promising new treatment modality for patients with inoperable, locally advanced or disseminated SS-R-positive tumours. The results of these experimental methods suggest that the anti-proliferative effect is due to selective binding to the SS-R and not to a systemic or secondary mechanism.

Therefore, we conclude that *(i)* high radioactive doses with [^{111}In-DTPA^{0}]octreotide for PRRT can inhibit the growth of SS-R-positive liver metastases in an animal model, *(ii)* PRRT is effective only if SS-Rs are present on the tumours and is, therefore, receptor-mediated and *(iii)* the effect of PRRT with [^{111}In-DTPA^{0}]octreotide can be reduced by pre-treatment with cold octreotide, which indicates that receptor binding is essentail for PRRT.

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